

APPLICANTS: Moutsatos I. et al.  
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FILED: September 4, 1998  
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cell expresses parathyroid hormone and a parathyroid hormone receptor protein.

### REMARKS

Claims 24-28 are pending in the Subject Application. Claims 11, 12, 14-17 and 19-23 have been previously cancelled, and claims 24, 25, 26 and 27 have been amended.

Applicants wish to thank the Examiner for his comments in the telephone conference of October 19, 2004, clarifying his position regarding the Advisory Action of October 12, 2004.

The Examiner noted that the wrong "status identifier" was used for claim 28. Applicants have, in the present response, addressed the "status identifier" objection raised by the Examiner, and submit that the Amendment presented herein conforms to accepted practice.

The Examiner maintained his allegation the term "organized" introduced in amended claims 24-28, changes the scope of the invention, necessitating a new search, and allegedly raising new 112 1<sup>st</sup> and 2<sup>nd</sup> paragraph issues. Applicants respectfully disagree.

The subject Application clearly provides literal support for the term "organized bone formation". On Page 25, lines 12-21 (please note underlined sections):

"Four weeks after transplantation, in non-union radial sites into which were transplanted C3H-BMP2 cells, we observed a unique regeneration process, which included well organized new growth of bone and cartilage *within the boundaries of the fracture edges*. In addition, *a collar of differentiating and calcifying chondrocytes was*

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*formed around the original edge of the bone defect....* In all of the other experimental groups, including the group that received collagen sponges carrying rhBMP-2 protein, any bone or cartilage observed was formed in a disorganized manner.”

Applicants submit that the phrase “well organized, new growth of bone” provides written description for the phrase “organized bone formation”. Further, Applicants contend that the examples by definition represent embodiments of the invention.

The Examiner has alleged that the “degree of organization” that satisfies the limitation of “organized functional bone formation” is unclear. Applicants disagree.

Applicants have clearly indicated on Page 25, italicized section in the paragraph cited above, that the level of organization is demonstrated by the examples provided. Organized bone formation occurs “within the boundaries of the fracture edges”. Further, on Page 26, lines 12-14, the degree of organization is described as:

“Following C3H-BMP-2 transplantation, bone and cartilage formed around the fracture edge appeared organized and oriented according to the original pattern of radial bone, thus better reconstructing its original structure”.

One skilled in the art would necessarily understand that the claimed “organized bone formation” occurs at a site of bone infirmity, in an orientation according to the original pattern of radial bone, to better reconstruct its original structure, which produced the resulting functional bone.

Moreover, the term “organized” is an art recognized term. Applicants submit that the term “organized”, as defined in the Merriam-Webster’s Dictionary (10<sup>th</sup> Edition, at page 819, the excerpt of which is attached hereto as Appendix 1), is “to cause to develop an organic structure, or to form into a coherent unity or functioning whole”. Applicants have clearly demonstrated the formation of a coherent unity and functioning whole, in the bone formed as

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a result of implantation of ex-vivo cultured, MSC engineered to express BMP-2 (as described hereinabove, in reference to the excerpts from pages 25 and 26, *supra*).

The Examiner has alleged that the inclusion of the term "organized" changes the scope of the invention and necessitates a new search. Applicants respectfully disagree. Applicants submit that the term "organized" does not broaden the scope of the claims, and thus does not necessitate a new search.

Applicants also wish the Examiner to consider the following arguments (also set forth by the Applicants in a September 27, 2004 response to the June 15, 2004 Office Action, issued by the USPTO, which was not admitted by the Examiner):

**REJECTION UNDER 35 U.S.C. 112:**

In the Office Action, the Examiner alleged that the subject matter does not reasonably convey to one skilled in the art that the inventor at the time the Application was filed was in possession of the invention. In particular, the rejection was based on claims directed to mesenchymal stem cells.

Applicants respectfully disagree. Example 11 of the subject Application clearly demonstrates the successful use of ex-vivo C3H10T1/2 cells transformed/transduced with BMP-2, for implantation at a site of a bone infirmity, resulting in the formation of organized, functional, bone formation at the defect site, in particular along the defect edges. Applicants maintain that one skilled in the art would know and understand that C3H10T1/2 cells are representative of mesenchymal stem cells, and Applicants maintain the following references support that such knowledge was in possession of one skilled in the art, at the time of the filing of the Application. The references are:

1) Nakamura T, Aikawa T, Iwamoto-Enomoto M, Iwamoto M, Higuchi Y, Pacifici M, Kinto N, Yamaguchi A, Noji S, Kurisu K, Matsuya T, Maurizio P. Induction of osteogenic differentiation by hedgehog proteins. *Biochem Biophys Res Commun.* 1997 Aug 18; 237(2):465-9.

2) Ahrens M, Ankenbauer T, Schroder D, Hollnagel A, Mayer H, Gross G. Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into

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distinct mesenchymal cell lineages. DNA Cell Biol. 1993 Dec;  
12(10):871-80.

Thus, contrary to the Examiner's assertion, there is support in the specification as filed for the term "mesenchymal stem cell"

**REJECTION UNDER 35 U.S.C. § 103:**

In the Office Action, the Examiner rejected claims 24-26 under 35 U.S.C. § 103 as allegedly being unpatentable over Ahrens et al, in view of US Patent No. 5,763,416 and US Patent No. 6,048,964. The Examiner asserted that based on Ahrens et al, in view of US Patent No. 5,763,416 and US Patent No. 6,048,964, it would have allegedly been obvious to one of ordinary skill in the art to combine to make the claimed invention, that of preparing ex-vivo cultured stem cells transformed with BMP-2 for implantation at a site of a bone infirmity.

In response, Applicants traverse the rejection of claims 24-26 under 35 U.S.C. § 103. Applicants maintain that Ahrens et al, in view of US Patent No. 5,763,416 and US Patent No. 6,048,964, do not render the claimed invention obvious, nor would a person of ordinary skill in the art have had a reasonable and/or credible expectation of success in obtaining the instant claimed invention given the teachings of Ahrens et al, in view of US Patent No. 5,763,416 and US Patent No. 6,048,964. The Examiner has alleged that Bonadio discloses the use of bone progenitor cells transformed with a BMP for stimulating bone formation.

Applicants respectfully disagree. Applicants maintain that although Bonadio suggest the use of bone progenitor cells for stimulating bone formation, such a suggestion is merely speculative and not credible, in view of what Bonadio demonstrated, and the knowledge in the art at the time.

Bonadio demonstrates **only** direct gene transfer of a bone morphogenetic protein. Bondaio asserts that the constructs are targeted to progenitor cells, however, there is no

credible support for such a contention, nor is it likely that direct transfer of a nucleic acid to a site of bone infirmity is **appreciably** taken up *in situ* by a bone progenitor cell. Applicants submit in a Declaration herein (attached hereto as Appendix 2) that Bonadio **describes** the use of an adenoviral vector for gene transfer experiments for *in vivo* bone regeneration, however adenoviral vector uptake is mediated by the CAR receptor, whose expression has been shown to be drastically diminished, if not absent in hematopoietic progenitor cells, as compared to their differentiated counterparts. Hematopoietic progenitor cells in fact, are more differentiated than mesenchymal stem cells, and therefore it is unlikely that an even less differentiated cell type will exhibit appreciable CAR expression. The Rebel et al article, as described in the Declaration, further indicates that gene transfer does not appreciably occur in cells, which have diminished CAR expression. Thus, in the absence of a demonstration to the contrary, one skilled in the art, would assume, based on the foregoing, that adenoviral transfer of a gene would not be successful in progenitor cells *in situ*.

Bonadio further describes the use of DNA-soaked sponges as another means of gene delivery, wherein the DNA is purportedly taken up by progenitor cells. Applicants submit, as described in the attached Declaration, that less differentiated cells have less propensity toward DNA uptake, similar to adenoviral uptake, and therefore neither method described by Bonadio provides for appreciable uptake of a foreign DNA sequence by mesenchymal stem cells. Thus, one skilled in the art would not credibly believe that Bonadio could predict uptake of a construct *in situ* by progenitor or stem cells.

Further, the Examiner has alleged that the motivation to combine the Bonadio and Ahrens references need only take into account a reasonable expectation of success in treating a site of bone infirmity in a human through the use of cultured mesenchymal stem cells that overexpress BMP-2, and the fact that Applicants data demonstrates the presence of autocrine and paracrine effects such cells demonstrates the fact that these mechanisms are necessarily present. Applicants respectfully disagree. Applicants maintain that there is no motivation to combine these references with a reasonable expectation of success for inducing organized, functional bone formation **at a site of bone infirmity** in a human.

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Though Bonadio describes that progenitor cells are targeted by his gene transfer methods, such a conclusion is not credible, in lieu of direct demonstration by Bonadio that such is the case, as much of the cell population targeted is not a stem or progenitor cell, moreover, uptake of the DNA by such cells *in situ*, according to one skilled in the art is drastically reduced, such that Bonadio does not credibly provide a foundation that BMP gene transfer provides more than paracrine effects for healing a bone infirmity.

While Ahrens provides for *in vitro* responses of progenitor cells to **a group of osteoinductive compounds**, which include *inter-alia*, a BMP, Ahrens provides no basis for the likelihood that implantation of such cells, transduced only with a BMP-2 vector, *in vivo* will stimulate bone induction **at a site of bone infirmity**. Such a result is predicated on appropriate cell homing and orientation along the defect edges, a result, which could not have been foreseen, based on either Ahrens, or credibly considered, in view of Bonadio. Moreover, the combination of Bonadio and Ahrens could not have predicted the unexpected results of the claimed invention, the formation of organized, functional bone formation evidenced in the instant invention, nor do they render obvious the likelihood of such formation at a site of bone infirmity.

Further, Ahrens demonstrates differentiation of MSCs *in vitro*, and in fact, as described in the Declaration attached hereto (Appendix 2), mesenchymal stem cells, which are cultured and differentiated *in vitro* when implanted *in vivo*, do not form functional tissue, and lose their cell surface marker phenotype (De Bari C. et al., Arthritis Rheum. 2004 Jan; 50(1): 142-50). Thus, in view of the art cited, Ahrens in combination with Bonadio do not credibly suggest that an *ex-vivo* cultured, BMP, much less a BMP-2 transduced/transformed mesenchymal stem cell will form organized, functional bone at a site of bone infirmity following implantation.

Applicants maintain that the presence of autocrine and paracrine effects of expressed bone morphogenesis protein 2 resulted in the organized, functional bone formation **at the site of bone infirmity**. Applicants maintain that it would not be obvious to combine the teachings of Bonadio, which only credibly describes paracrine effects of BMP on bone formation alone, and Ahrens, which demonstrates autocrine effects of **a group of osteoinductive compounds**

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to arrive at enhanced, organized, functional bone formation at a site of bone infirmity. Applicants maintain that combining the references of Bonadio and Ahrens do not credibly suggest cultured, progenitor cells transformed/transduced with a BMP-2 alone, would effectively form bone at a site of infirmity, much less, that such bone formed would be enhanced, and organized along defect edges, and providing functional bone, which could only be revealed by the claimed invention.

Moreover, Applicant's unexpectedly discovered that *ex-vivo* cultured mesenchymal stem cells transduced/transformed with BMP such as BMP-2 form greater amounts of bone, than what is achieved by direct gene transfer, the presence of the protein, or differentiated cells secreting BMP-2, and that the bone formation is oriented along defect edges. Applicants results demonstrate bone formation, qualitatively and quantitatively, via the implantation of *ex-vivo* cultured, BMP-2 transduced/transformed mesenchymal stem cells, at a site of bone infirmity. Thus, a method of inducing organized (as exemplified in the subject Application in Example 11, by orientation of the bone formation along defect edges), bone formation at a site of bone infirmity, is novel and unobvious in view of the art.

In addition, the Examiner has also rejected claim 27 in view of the above cited references, further in view of Wozney, under 35 USC 103.

Wozney describes expression of a BMP receptor for BMP-2 in cells responding to the growth factor. Applicants maintain, that since neither Bonadio nor Ahrens provide a credible basis for the use of *ex-vivo* cultured MSC transduced/transformed with BMP-2 alone, in inducing organized functional bone formation at a site of bone infirmity, then the engineering of such cells to further express a BMP receptor is not rendered obvious, in consideration of Wozney.

Bonadio **describes** the use of an adenoviral vector for gene transfer experiments for *in vivo* bone regeneration, however adenoviral vector uptake is mediated by the CAR receptor, whose expression has been shown to be drastically diminished, if not absent in hematopoietic progenitor cells, as compared to their differentiated counterparts. Ahrens provides for *in vitro* responses of progenitor cells to **a group of osteoinductive compounds**, which include *inter-*

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*alia*, a BMP, Ahrens provides no basis for the likelihood that implantation of such cells, transduced only with a BMP-2 vector, *in vivo* will stimulate bone induction **at a site of bone infirmity**. Moreover, the combination of Bonadio and Ahrens could not have predicted the unexpected results of the claimed invention, the formation of organized, functional bone formation evidenced in the instant invention, nor do they render obvious the likelihood of such formation at a site of bone infirmity.

Thus, since Bonadio and Ahrens do not render obvious the methods of inducing functional bone formation via implanting ex-vivo cultured MSC transfected with BMP-2, Applicants maintain that Wozney, further in view of the two does not render obvious the engineering of such cells to further express a BMP receptor.

In addition the Examiner rejected claim 28 in view of the above cited references, further in view of Hattersley, under 35 USC 103. Hattersley describes the use of PTH and its receptor in the context of BMP-2. Applicants maintain, that Bonadio and Ahrens do not render obvious the methods of inducing functional bone formation via implanting ex-vivo cultured MSC transfected/transduced with BMP-2, and since the methods are not obvious in view of the art, neither is MSC expression of a PTH/PTH receptor. Therefore, Applicants submit that the additional reference does not render the instant invention obvious.

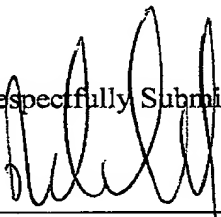
Accordingly, Applicants request the Examiner to reconsider and withdraw the rejection of the claims under 35 U.S.C. 103.

Accordingly, Applicants submit that the pending claims are allowable, and that Applicants have addressed all prior Rejections. Their favorable reconsideration and allowance is respectfully requested. Should the Examiner have any question or comment as to the form, content or entry of this Amendment, the Examiner is requested to contact the undersigned at the telephone number below.



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Should any fee be due, the undersigned Attorney hereby authorizes the United States Patent and Trademark Office to charge Deposit Account No. 50-3355 for any fees required.

Respectfully Submitted,  
  
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## dinary-language philosophy • organum 819

pounds of living beings and most other carbon compounds 4 a : forming an integral element of a whole : FUNDAMENTAL (incidental music rather than ~ parts of the action —Francis Fergusson) b : having systematic coordination of parts : ORGANIZED (an ~ whole) c : having the characteristics of an organism : developing in the manner of a living plant or animal (society is ~) 5 : of, relating to, or constituting the law by which a government or organization exists — **or-gan-i-cal-ly** \-ni-k(ə)-lē\ *adv* — **or-ga-nic-i-ty** \,ör-gə-'ni-sə-tē\ *n*

**organic** *n* (1942) : an organic substance: as a : a fertilizer of plant or animal origin b : a pesticide whose active component is an organic compound or a mixture of organic compounds

**or-gan-i-cism** \ör-'ga-nə-'si-zəm\ *n* [ISV] (1883) 1 a : the explanation of life and living processes in terms of the levels of organization of living systems rather than in terms of the properties of their smallest components b : VITALISM 2 : any of various theories that attribute to society or the universe as a whole an existence or characteristics analogous to those of a biological organism — **or-gan-i-cist** \-sist\ *n* or *adj*

**or-ga-ni-sa-tion**, **or-ga-nise**, **or-ga-nis-er** *Brit var of ORGANIZATION*, **ORGANIZE**, **ORGANIZER**

**or-gan-ism** \'ör-gə-'ni-zəm\ *n* (ca. 1774) 1 : a complex structure of interdependent and subordinate elements whose relations and properties are largely determined by their function in the whole 2 : an individual constituted to carry on the activities of life by means of organs separate in function but mutually dependent : a living being — **or-gan-is-mic** \,ör-gə-'niz-mik\ *also* **or-gan-is-mal** \-mə\ *adj* — **or-gan-is-mi-cal-ly** \-mi-k(ə)-lē\ *adv*

**or-gan-ist** \'ör-gə-'nist\ *n* (1591) : a person who plays the organ

**or-ga-ni-za-tion** \,ör-gə-nə-'zā-shən, ,ör-gə-nə\ *n* (15c) 1 a : the act or process of organizing or of being organized b : the condition or manner of being organized 2 a : ASSOCIATION, SOCIETY (charitable ~s) b : an administrative and functional structure (as a business or a political party); *also* : the personnel of such a structure

**organization** *adj* (1949) : characterized by complete conformity to the standards and requirements of an organization (an ~ man)

**or-ga-ni-za-tion-al** \-shnəl, -shə-n'ē\ *adj* (1881) 1 : of or relating to an organization : involving organization (the ~ state of a crystal) 2 : ORGANIZATION — **or-ga-ni-za-tion-al-ly** *adv*

**or-ga-nize** \'ör-gə-'nīz\ *vb* -nized; -niz-ing *vt* (15c) 1 : to cause to develop an organic structure 2 : to form into a coherent unity or functioning whole : INTEGRATE (trying to ~ her thoughts) 3 a : to set up an administrative structure for b : to persuade to associate in an organization; *esp* : UNIONIZE 4 : to arrange by systematic planning and united effort ~ *vi* 1 : to undergo physical or organic organization 2 : to arrange elements into a whole of interdependent parts 3 : to form an organization; *specif* : to form or persuade workers to join a union *syn* see ORDER — **or-gan-iz-able** \,ör-gə-'nī-zə-bəl\ *adj*



Attorney Docket No. P-4739-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Moutsatsos et al. Examiner: Sandals W.  
Serial No.: 09/148,234 Group Art Unit: 1636  
Filed: September 4, 1998  
Title: GENETICALLY ENGINEERED CELLS WHICH EXPRESS BONE  
MORPHOGENIC PROTEINS

DECLARATION UNDER RULE 37 C.F.R. 1.132

Assistant Commissioner for Patents  
Washington, DC 20231

I, Dan Gazit, a citizen of Israel, residing at 46 Perez Berenstein Street, Jerusalem, 96920,  
hereby declare:

1. I am a Professor director the Biotechnology centre at the Hebrew University- of Jerusalem. I have a Ph.D. in bone biology from the Hebrew University, Jerusalem Israel. My fields of expertise are skeletal biotechnology and developmental molecular biology. Specifically I have been involved in the study of Adult Human Mesenchymal stem cells and Skeletal tissue engineering.
2. My Curriculum Vitae and list of publications are attached herewith as Appendix 1.
3. I have read the subject Application and have reviewed the patent Prosecution History, including the Office Action of June 15, 2004. The subject Application

describes *inter alia*, *ex-vivo* methods of transforming or transducing mesenchymal stem cells *in vitro* with a nucleic acid, which encodes for BMP-2 protein, for the implantation in a subject in need for bone repair or regeneration.

4. Claim 24 of the subject Application recites a method of inducing enhanced, organized, functional bone formation at a site of bone infirmity in a human, comprising the steps of:

- (a) transforming a cultured mesenchymal stem cell with a DNA encoding bone morphogenesis protein 2 (BMP-2);
- (b) culturing the cultured mesenchymal stem cell transformed in step (a), under conditions enabling expression of said DNA encoding bone morphogenesis protein 2; and
- (c) implanting said cultured mesenchymal stem cell at a site of bone infirmity

whereby autocrine and paracrine effects of expressed bone morphogenesis protein 2 at said site of bone infirmity result in enhanced, organized, functional bone formation, thereby inducing functional bone formation at a site of bone infirmity.

5. In the Office Action, the Examiner rejected the claims of the above-identified Application as allegedly being obvious to one skilled in the art, based on Ahrens et al. (DNA and Cell Biology, Volume 12, NO. 10, pages 871-880, 1993) and in view of United States Patent No. 5,763,416 (Bonadio et al.) and United States Patent No. 6,048,964. The Examiner asserted that Bonadio allegedly discloses a method of producing cultured or bone marrow stromal cells for implantation at the site of bone infirmity by transforming the cells with recombinant bone morphogenetic protein. Specifically, the Examiner asserted "the cited references comprise teachings that provide a reasonable expectation of success in treating a site of bone infirmity in a human through the use of cultured mesenchymal stem cells that overexpress BMP-2".

6. The Examiner stated that Bonadio describes the use of bone progenitor cells transformed with a BMP for stimulating bone formation, and their functioning via autocrine and paracrine effects is expected. Further, the Examiner

contended that the motivation to combine the Bonadio and Ahrens references need only take into account a reasonable expectation of success in treating a site of bone infirmity in a human through the use of cultured mesenchymal stem cells that overexpress BMP-2, and the fact that Applicants data demonstrates the presence of autocrine and paracrine effects such cells demonstrates the fact that these mechanisms are necessarily present.

7. It is my opinion that the Examiner is incorrect in his assertion. Bonadio does not provide a credible foundation for a method of stimulating bone formation at a site of a bone infirmity by implanting a mesenchymal stem cell transformed/transduced with a BMP-2 construct. Though Bonadio describes that progenitor cells are targeted by his gene transfer methods, such a conclusion is not credible, in lieu of a direct demonstration by Bonadio, since much of the cell population targeted by direct gene transfer is not a stem or progenitor cell, which represents a small population of cells *in vivo*, at a site of bone infirmity. Moreover, uptake of the DNA by such cells *in situ*, is known to one skilled in the art to be drastically reduced (see for example, Rebel V.I. et al., Stem Cells (2000) 18: 176-82; Zhao Q. et al., Blood (1994) 84:3660-6), such that Bonadio does not credibly provide a foundation that BMP gene transfer provides more than paracrine effects for healing a bone infirmity.
8. Ahrens discloses *in vitro* responses of progenitor cells to a group of osteoinductive compounds (which include, *inter-alia*, a BMP), Ahrens provides no basis for the likelihood that implantation of such cells, transduced only with a vector expressing a BMP, *in vivo*, will stimulate bone induction at a site of bone infirmity. Such a result is predicated on appropriate cell homing and orientation along the defect edges, a result, which could not have been foreseen, based on Ahrens.
9. Further, Ahrens demonstrates differentiation of MSCs *in vitro*, which studies show (De Bari C. et al., Arthritis Rheum. 2004 Jan; 50(1):142-50) when implanted *in vivo*, these MSCs do not form functional tissue, and lose their cell surface marker phenotype. Thus, in view of the art cited, Ahrens does not credibly teach an *exclusive* effect of BMP-2, nor for that matter does Ahrens

credibly provide for an exclusive effect of any BMP, on mesenchymal stem cell bone induction. One skilled in the art would not believe the MSCs of Ahrens to be able to induce enhanced, organized, functional bone, once implanted in vivo. The combination of Ahrens with Bonadio do not credibly suggest that an ex-vivo cultured, BMP-2 transduced/transformed mesenchymal stem cell will form enhanced, organized, functional bone at a site of bone infirmity following implantation. Certainly both references do not unequivocally demonstrate an effect of BMP-2 alone, on MSCs for stimulating bone induction, nor suggest their role in stimulating, enhanced, organized, functional bone induction, specifically at a site of a bone infirmity.

10. Accordingly, it is my opinion that there is also no motivation to combine these references with a reasonable expectation of success for inducing organized, functional bone formation at a site of bone infirmity in a human by implanting an ex-vivo cultured MSC transduced/transformed with any BMP, and in particular BMP-2. Both Bonadio and Ahrens disclosures do not produce a population of cells capable of forming organized, functional bone at a site of bone infirmity, the former, due to the improbability of obtaining such a cell, and the latter, due to the improbability of obtaining a cell that would function *in situ*, and the fact that there is no osteoinductive compound functioning alone defined.
11. The combination of Bonadio and Ahrens could not have reasonably predicted the unexpected results obtained in the claimed invention, which resulted in enhanced, organized, functional bone formation at a site of bone infirmity. In vivo studies (*Gazit et al.*, 1999, *J Gene Med* 1: 121-133, a copy of which is attached hereto as Appendix 3), demonstrated that engineered progenitor cells (C3H-BMP2), in comparison to administration of 3 µg recombinant human BMP2, or engineered non progenitor cells (CHO-BMP2) produced enhanced bone formation, and most surprisingly, that the formation was in alignment with the original defect edge, this despite the fact that greater amounts of BMP-2 were secreted from the CHO BMP-2 cells.



effects of BMP-2, as neither Bonadio nor Ahrens credibly describe targeting of progenitor cells *in situ* or lone effects of any BMP, in particular BMP-2. Thus, neither Ahrens, nor further in view of Bonadio credibly describe a means of providing the enhanced, organized, functional bone at a site of bone infirmity, as claimed in the instant invention.

13. In view of the reasons and the facts described above, one skilled in the art would not be able to predict the enhanced, organized, functional bone induction at a site of bone infirmity produced via implantation of *ex vivo* transformed/transduced MSCs with BMP-2, as claimed in the subject Application.

The undersigned further declares that all statements made herein of his own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 9/19/04 \_

*Dan Gazit*

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Dan Gazit



BIOGRAPHICAL SKETCH			
NAME Gazit Dan	POSITION TITLE Associate Professor		
EDUCATION/TRAINING	DEGREE	YEAR	FIELD OF STUDY
Hebrew University of Jerusalem	D.M.D.	1970-1976	Dental Surgeon
Hebrew University of Jerusalem	Ph.D.	1986-1991	Bone Biology

#### Professional Experience

1981-1985	Instructor in Oral Pathology, Oral Pathology, Hebrew University of Jerusalem
1986-1992	Lecturer in Oral Pathology, Bone Biology and Pathology, Hebrew University of Jerusalem
1990-1992	Visiting Professor, Bone Biology, UCSF
1992-1999	Tenured Senior Lecturer in Oral Pathology, Bone Biology, Biotechnology and Pathology, Hebrew University of Jerusalem
1996-2001	Director, Oral Pathology, Oral Pathology Biopsy Facility, The Hebrew University- Hadassah Faculty of Dental Medicine
1997-	Director, Hebrew University Dental Sciences Graduate Program
1999	Visiting Professor, Bone Biology, Leiden Medical Center
1999	Visiting Professor, Bone Biology, Boston University
1999	Visiting Professor, Bone Biology, Harvard Medical School
1999-	Associate Professor, Bone Biology and Biotechnology, Hebrew University of Jerusalem

#### Professional Membership

1999-	Gene Therapy Steering Committee Member, Gene Therapy Center, Hadassah Medical Center, Jerusalem
2002-	Chairman of Biotech Committee, Hebrew University of Jerusalem

#### Selected peer-reviewed publications

1. Gazit, D., Zilberman, Y., Ebner, R. and Kahn, A. (1998) Evidence that bone loss (osteopenia) in old, male mice results from the diminished activity and availability of TGF- $\beta$ 1. J. Cell. Biochem. 70:478-488.
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# Maturation and Lineage-Specific Expression of the Coxsackie and Adenovirus Receptor in Hematopoietic Cells

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## ABSTRACT

Adenovirus vectors have been used to transfer genes into both hematopoietic progenitor cells and tumor cells, including carcinoma cells that have metastasized to bone marrow (BM). However, the relative susceptibility of different subsets of hematopoietic cells is unknown. In permissive cells adenoviral-mediated gene transfer is mediated by the coxsackievirus and adenovirus receptor (CAR) protein and  $\alpha$ , integrins expressed on the cell surface of the target cells. This prompted us to investigate the expression of CAR on subpopulations of hematopoietic cells, determine whether this protein played a role in adenovirus-mediated gene transfer of hematopoietic cells and whether we could modulate CAR to enhance

gene transfer efficiency. In this report we show that CAR is expressed on approximately 40% of all human BM cells, including erythroid and myeloid cells, but not lymphoid cells. Of the CD34<sup>+</sup> cells, 10%-15% expressed CAR, but this did not include most colony-forming progenitor cells, nor the most primitive CD38<sup>-</sup> subpopulation. The presence of CAR correlated well with gene transfer efficiency, but we were unable to induce CAR expression on immature, noncommitted progenitor cells. In conclusion, our results show that primitive hematopoietic progenitor cells lack CAR expression, but that expression is acquired during erythroid and myeloid differentiation. *Stem Cells* 2000;18:176-182

## INTRODUCTION

The easy accessibility of hematopoietic progenitor cells and their ability to generate long-term progeny *in vivo* are two characteristics that make these cells important targets for gene therapy. For this purpose, a wide variety of viruses have been used including retro-, adeno-, adeno-associated, and lentiviruses [1-6]. Adenoviruses are able to infect noncycling cells and can be concentrated to extremely high titers; however, gene expression is transient. Thus, for gene therapy applications in which transient gene expression is desired, adenovirus may be the preferred vector for gene delivery into quiescent hematopoietic progenitor cells. Examples are the delivery of the amphotropic retroviral receptor or a mitogen to increase the sensitivity of cells to subsequent retroviral infection or improve the success rate of integration of a retrovirus-encoded transgene into the genome, respectively

[7, 8]. The susceptibility of CD34<sup>+</sup> hematopoietic progenitor cells to adenovirus is somewhat controversial. Recent studies suggest that adenovirus vectors carrying a "suicide" gene may be suitable for bone marrow (BM) purging of cancer cells; in these experiments the breast carcinoma cells tested were much more easily transduced than freshly isolated BM cells, which were relatively resistant [9, 10]. Because of interest in using adenovirus as a purging vector, it is extremely important to establish the susceptibility of primitive BM cells to infection and the different mechanisms by which virus may enter the cell.

Cellular infection by adenovirus is a multistep process that involves the interaction of the trimeric fiber protein and the pentameric penton base protein of the virus with specific receptors on the target cells. First, the virus attaches to the cell, a process mediated by the fiber protein. The cellular

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receptor for the fiber protein was recently identified with the isolation of the common coxsackie and adenovirus receptor (CAR) protein [11]. After attachment, virus internalization and membrane permeabilization occur through the interaction of the penton base protein with  $\alpha_v$  integrins on the target cells [12]. Although adenovirus infection is most efficient when both CAR and  $\alpha_v$  integrins are present on the target cells, there is increasing evidence for successful adenovirus-mediated gene transfer using alternative pathways that circumvent the lack of either type of receptor [13, 14].

Adenovirus infection of human CD34<sup>+</sup> hematopoietic progenitor cells, a population that includes long-term repopulating stem cells, requires certain culture conditions and a high multiplicity of infectious (MOI) particles per cell [2]. Compared to certain primary cells or tumor cell lines, the relatively inefficient adenovirus-mediated gene transfer of hematopoietic progenitor cells may be in part due to the lack of expression of  $\alpha_v$  integrins on their cell surface [15, 16]. Little is known about the expression of CAR on hematopoietic cells, although mRNA for the CAR protein has been demonstrated in CD34<sup>+</sup> cells isolated from leukopheresis products [17]. In light of these findings, we wanted to investigate whether the CAR protein is expressed on the cell surface of subpopulations of hematopoietic cells. If so, we were interested in answering the following questions: A) does the expression of the CAR protein correlate with susceptibility to adenoviral gene transfer in hematopoietic cells and B) can we identify cytokines that modulate the expression of CAR and therefore the gene transfer efficiency? We show that CAR expression on freshly isolated BM cells is mainly found on differentiated erythroid and myeloid cells, on a small proportion of CD34<sup>+</sup> progenitor cells, but not on lymphoid cells. Gene delivery into freshly isolated CD34<sup>+</sup> cells correlates well with the level of CAR expression, but still requires large amounts of virus.

## MATERIALS AND METHODS

### Preparation of Human BM Cells

Discarded bags and attached filters from BM harvests of normal donors were rinsed with Iscove's modified Dulbecco's medium (IMDM) (Life Technologies; Grand Island, NY; <http://www.lifetech.com>) containing 2% fetal bovine serum (FBS) (Sigma Chemical Co.; St. Louis, MO; <http://www.sigma-aldrich.com>) to obtain the remaining BM cells. The cells were then centrifuged over a layer of Histopaque<sup>®</sup>-1077 (Sigma) to deplete erythroid and granulocytic cells. The cells were frozen (in IMDM, 50% FBS, 10% dimethylsulfoxide [Sigma]) and further separated on the day of the experiment. CD34<sup>+</sup> progenitor cells were enriched using a positive selection method as recommended by the

manufacturer (Ceptra L.C. separation system, CellPro; Bothell, WA).

### Cell Staining and Sorting

Flow cytometric detection of CAR on the cell surface was performed using the monoclonal antibody (mAb) RmcB [18], which was either directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin. To define the different hematopoietic subpopulations and their expression of CAR, BM cells were simultaneously stained with anti-CD34-cyanine 5 (Becton Dickinson; San Jose, CA; <http://www.bd.com>), RmcB-FITC, and a mAb directed against one of the following lineage markers: CD33, CD14, or CD38 (Becton Dickinson), glycophorin-A, CD4 together with CD8, or CD19 (PharMingen; San Diego, CA; <http://www.pharMingen.com>). In every experiment irrelevant isotype controlled mAbs were used to determine background staining. All staining procedures were done in phosphate buffered saline (PBS) (Life Technologies) that contained 2% FBS. The cell labeling was performed on ice (35 min) after which the cells were washed twice Propidium iodide (PI) (Sigma) (2  $\mu$ g/ml) was added during the second wash prior to resuspension in PBS, 2% FBS. Three-color flow cytometric analysis and cell sorting were performed on a Coulter Epics<sup>®</sup> Elite ESP (Coulter; Hialeah, FL; <http://beckmancoulter.com>).

To analyze individual colonies for CAR expression, colonies were plucked from methylcellulose (MC), incubated for 1 h in PBS containing 2% FCS, to allow the MC to dissolve, spun down once and subsequently stained with the appropriate mAbs. Two-color fluorescence-activated cell sorter (FACS) analysis of the MC colonies and the suspension cultures (see later) were analyzed on a single laser FACScan (Becton Dickinson; Mountain View, CA).

### Colony-Forming Cell (CFC) Assay

To determine the CFC content of the sorted CD34<sup>+</sup> BM cells, cells were plated in IMDM/0.9% MC media (Methocel MC, Fluka; Buchs, Switzerland; <http://www.sigma-aldrich.com>) containing 30% defined FBS (HyClone Laboratories Inc.; Logan, UT; <http://www.hyclone.com>) and the following human recombinant cytokines: Steel factor (SF) 50 ng/ml, interleukin 3 (IL-3) 20 ng/ml, GM-CSF (20 ng/ml), and erythropoietin (Epo) 3 U/ml. IL-3, IL-6, and GM-CSF were generous gifts from Genetics Institute (Cambridge, MA; <http://www.genetics.com>). SF and Epo were purchased from R&D Systems (Minneapolis, MN; <http://www.rndsystems.com>). Duplicates of 1,000 cells (or as otherwise indicated) per 35 mm dish were plated. Colonies were scored in situ after 14–20 days of incubation at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air using well-established criteria [19].

### Serum-Free Suspension Cultures

Enriched CD34<sup>+</sup> BM cells were cultured in serum-free medium prepared as described previously [20]. Cells were initially cultured in 1 ml volumes in 24-well culture plates and kept at a density below  $1 \times 10^6$  cells/ml. The medium was supplemented with various combinations of the following cytokines: SF (50 ng/ml), Flt-3 ligand ([FL] 100 ng/ml), IL-6 (10 ng/ml), IL-3 (20 ng/ml), IL-11 (25 ng/ml), GM-CSF (20 ng/ml), and Epo (2 U/ml). FL was kindly provided by Immunex (Seattle, WA; <http://www.immunex.com>) and IL-11 by Genetics Institute. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. At subsequent days, the cultures were harvested, viable cells (excluding trypan blue) were counted using a hemocytometer and phenotypic analysis was performed as described above.

### Adenovirus Construction and Preparation

The adenovirus vector that contains the green fluorescent protein gene (AdGFP) was kindly provided by Bob Carter and Richard Mulligan (Howard Hughes Medical Institution, Children's Hospital, Boston, MA; <http://www.hhmi.org>), and was constructed by first subcloning the GFP cDNA into pAdlox, a shuttle vector that contains a single loxP site. This expression cassette was linearized and cotransfected into CRE8 cells with the  $\psi$ 5 helper virus, which is an E1- and E3-deleted version of Ad5 that contains loxP sites flanking the packaging site. Recombination occurs between the two linear molecules at the loxP sites [21]. We then plaque-purified the virus and expanded it on 293 cells using standard techniques. Each virus inoculum was purified by a CsCl step gradient followed by a CsCl equilibrium gradient, dialyzed against a glycerol buffer and stored at -20°C.

### Adenoviral Infection Protocol and Analysis

After a short culture period (~4 h) CD34<sup>+</sup>-enriched cells were incubated with AdGFP for 20 h at 37°C, at a MOI of 500, or otherwise indicated. The incubations were done in serum-free medium, supplemented with SF, FL, IL-6, and Epo in 100-200  $\mu$ l volumes in 96-well plates when  $<10^5$  cells were to be infected, or in 1  $\mu$ l cultures in 24-well plates when the cell number was between  $10^5$ - $10^6$ . The analysis by FACS for green fluorescence intensity as a measure for gene transfer was performed immediately after the 24 h of culture.

## RESULTS

### Expression of CAR on the Cell Surface of Subpopulations of Hematopoietic Cells

BM cell suspensions were stained with mAbs directed against CAR and various lineage markers representative for

erythroid (glycophorin-A), myeloid (CD33 and CD14), and lymphoid (CD19 and CD4/CD8) cells. Figure 1 shows representative FACS profiles: CAR is expressed on ~40% of total BM cells, including glycophorin-A<sup>+</sup> cells, CD14<sup>+</sup>, and CD33<sup>+</sup> cells. In contrast, very few CAR<sup>+</sup> cells can be demonstrated among the lymphoid CD19<sup>+</sup> or CD4/8<sup>+</sup> cells. To identify CAR expression on more primitive hematopoietic progenitor cells, BM cells were stained with a cocktail of mAbs identifying CD34, CD38, and CAR. From Figure 2A it is immediately clear that the level of CAR expression on CD34<sup>+</sup> cells is considerably lower than that on mature myeloid or erythroid cells (Fig. 1). Only 10%-15% of CD34<sup>+</sup> cells express CAR at a level comparable to that of, e.g., CAR<sup>+</sup>CD33<sup>+</sup> cells (box 2, Fig. 2A) and 1%-2% express high levels of CAR (box 1, Fig. 2A). Counterstaining CD34<sup>+</sup>CAR<sup>+</sup> cells with a combination of lineage markers (glycophorin-A, CD14, CD33, CD38, CD19, CD4, and CD8) revealed that these cells expressed one or more of these markers (data not shown). This finding suggests that CAR expression on hematopoietic BM cells may be limited mostly to mature erythroid and

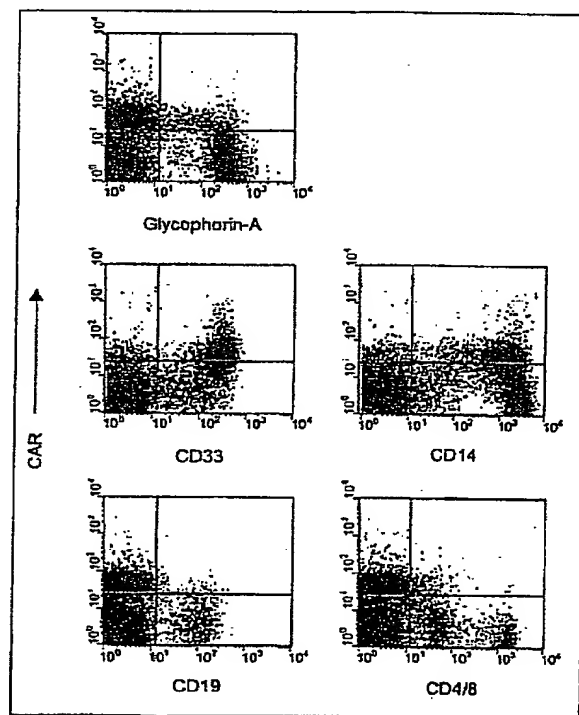


Figure 1. CAR is expressed on the cell surface of erythroid and myeloid, but not lymphoid cells. Depicted are representative profiles of live (PI<sup>-</sup>) unseparated BM mononuclear cells.

myeloid cells and a small proportion of committed progenitor cells. Indeed, when CAR expression was determined on CD34<sup>+</sup>CD38<sup>-</sup> cells, a population of cells that contains primitive nonobese diabetic/severe combined immunodeficiency (NOD/SCID) repopulating cells [22, 23], CAR expression was not detectable (Figs. 2B and 2C).

#### CAR Expression on CFC

The phenotypic analysis suggested that the majority of the CD34<sup>+</sup> progenitor cells does not express CAR. We wanted to investigate whether functional analysis could validate this result. CD34<sup>+</sup> BM cells were separated on the basis of CAR expression

as indicated in Figure 2A (box 1-3) and the different subsets were then analyzed for their ability to form colonies in MC. One such analysis is shown in Table 1. Most of the colonies are recovered in the CAR<sup>-</sup> fraction, a distribution that is in accordance with the relative CAR expression on CD34<sup>+</sup> cells.

Since the progeny of CFC are more differentiated cells, we were interested to determine the CAR expression on these cells. Individual colonies from the CAR<sup>-</sup> fraction were therefore isolated and the cells stained with an anti-CAR mAb together with the appropriate lineage marker to confirm the morphological appearance of the colony. Colonies scored as BFU-E were counterstained with glycophorin-A and colonies

**Figure 2.** CD34<sup>+</sup>CD38<sup>-</sup> progenitor cells do not express CAR. CD34<sup>+</sup>-selected cells were stained with mAb directed against the indicated cell surface antigens. Shown are representative FACS profiles from live (PI<sup>-</sup>) cells. A. In this experiment 11.4% of all CD34<sup>+</sup> cells expressed intermediate levels of CAR (box 2) and 1.2% high levels (box 1). B. Simultaneous staining with antiCD34 and antiCD38 mAbs revealed that the CD34<sup>+</sup>CD38<sup>-</sup> cells (indicated by the cells in box 4) do not express CAR (C).

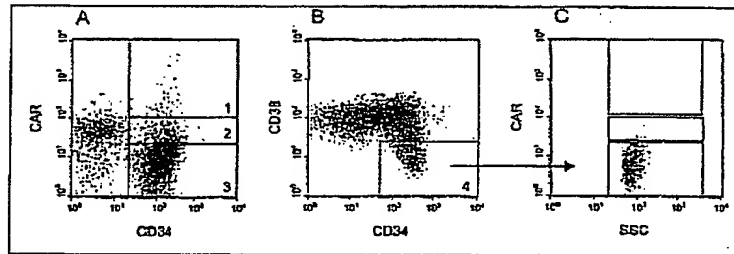


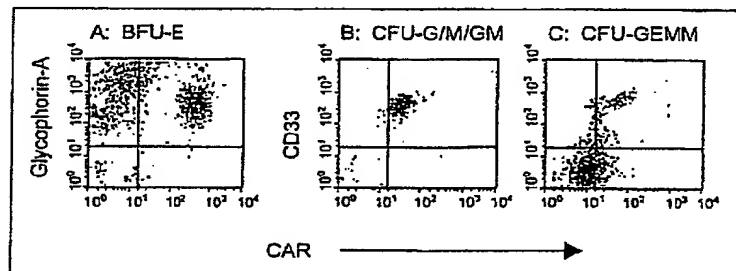
Table 1. The majority of CFC does not express CAR							
CD34 <sup>+</sup> subset	Fraction of all CD34 <sup>+</sup> cells (%)	Number of Colonies (per 10 <sup>4</sup> cells)			Recovery of Colonies (%)		
		BFU-E	G/M/GM	GEMM	BFU-E	G/M/GM	GEMM
CAR <sup>-</sup>	85.1	600	450	110	83	78	93
CAR <sup>int</sup>	11.4	660	750	50	12	18	6
CAR <sup>hi</sup>	3.5	767	600	33	4	4	1

Data represent one of two experiments; the other experiment showed very similar results

\*CD34<sup>+</sup> cells were sorted as indicated in Figure 2A (boxes 1-3). Duplicates of 1,000 cells per dish were plated

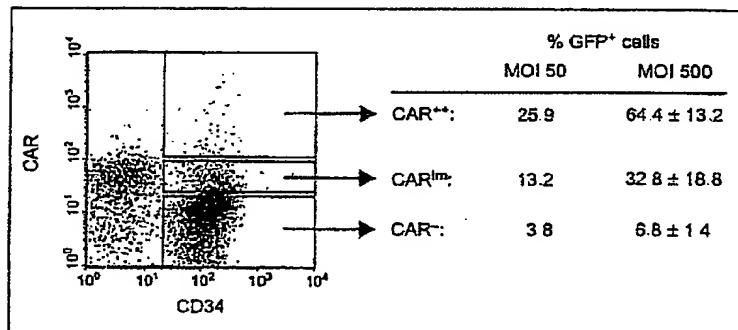
<sup>b</sup>The recovery was calculated as follows: for each subset and type of colony, the observed number per 10<sup>4</sup> cells was multiplied by the respective fraction that the subset represented of the total CD34<sup>+</sup> cells. This corrected number was then divided by the total number of colonies recovered (i.e., the sum of the corrected colony numbers of the three subsets) and multiplied by 100.

**Figure 3.** CAR<sup>+</sup> cells can be found among the progeny of all types of CFC. Shown are representative FACS profiles of live (PI<sup>-</sup>) cells obtained by plucking MC colonies 14 days after the cells had been plated. Erythroid colonies (A) mostly showed a profile as presented here, but occasionally a level of CAR that was comparable to that of cells obtained from CFU-G/M/GM colonies (B) was detected. (C) Mixed lineage colonies contained cells from the erythroid, myeloid and megakaryocytic lineage





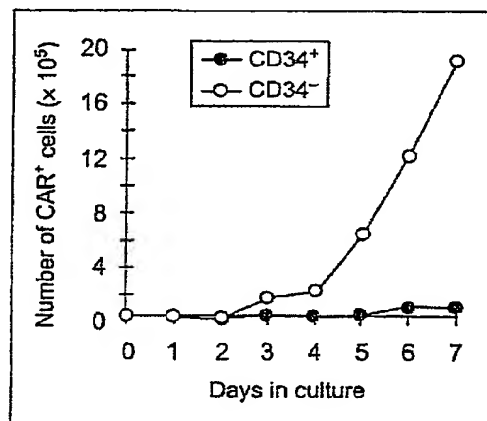
**Figure 4. Expression of CAR correlates with adenovirus-mediated gene transfer efficiency.** Pre-enriched CD34<sup>+</sup> cells were simultaneously stained with anti-CAR and anti-CD34 mAbs. CD34<sup>+</sup> cells were sorted on the basis of CAR as indicated by the boxes. The results of 20 h exposure to AdGFP during a 24-h culture period for each fraction are shown in the figure. Each data point with MOI 500 consists of two to three independent experiments. The data points obtained with MOI 50 represent a single experiment. CAR<sup>hi</sup> cells are CD34<sup>+</sup> cells that express intermediate levels of CAR. im = intermediate



scored as granulocytic and/or monocytic (CFU-G/M/GM) were counterstained with CD33. The multilineage colonies (CFU-GEMM) were also stained with CD33 to identify the myeloid component in an often dominant erythroid appearance. Figure 3 shows the various types of colonies that were identified by flow cytometry. A large proportion of cells isolated from erythroid colonies stained brightly positive for CAR (Fig. 3A). Cells from myeloid colonies all stained intermediate positive for CAR (Fig. 3B), as did the CFU-GEMM, but the level of CAR expression was on average lower than that of the myeloid colonies (Fig. 3C). Thus, while the majority of clonogenic progenitors is CAR<sup>+</sup> (Table 1), their progeny show an increase in the level of CAR expression (Fig. 3).

#### Adenoviral Gene Transfer Efficiency in Relation to CAR Expression

To determine whether there was a correlation between CAR expression and efficiency of adenoviral gene transfer in CD34<sup>+</sup> cells, CD34<sup>+</sup> cells were separated on the basis of CAR expression, and the different fractions were cultured for 24 h. During the last 20 h of culture, cells were exposed to an adenovirus construct that contained the gene for the AdGFP. After 24 h, the culture was then analyzed by FACS for GFP expression (i.e., green fluorescence intensity). Figure 4 shows the combined results of three such experiments. The best gene transfer efficiency was indeed obtained with cells that expressed the highest level of CAR (CAR<sup>hi</sup> cells); 64.4 ± 13.2%, compared to 6.8 ± 1.4% in cells that did not express CAR (CAR<sup>lo</sup> cells). The cells that expressed intermediate levels of CAR (CAR<sup>im</sup> cells) showed intermediate levels of gene transfer: 32.8 ± 18.8%. This effect was dose-dependent; decreasing the MOI 10-fold reduced the gene transfer rate considerably. Thus, the level of CAR expression on freshly isolated CD34<sup>+</sup> BM cells correlates well with the proportion of GFP<sup>+</sup> cells after a 24-h exposure to AdGFP.



**Figure 5. CAR<sup>+</sup> cells produced in culture are mostly CD34<sup>+</sup>.** Presented is one of three experiments, showing the number of CAR<sup>+</sup> cells (CD34<sup>+</sup> and CD34<sup>-</sup>) that initiated the culture at day 0 and the production of CAR<sup>+</sup> cells (CD34<sup>+</sup> and CD34<sup>-</sup>) at subsequent days. The number of cells was calculated by multiplying the total cell number by the fraction of cells of a particular phenotype obtained by FACS analysis.

#### Cytokines Do Not Induce CAR Expression

BM cells enriched for CD34<sup>+</sup> progenitor cells were cultured under serum-free conditions to determine whether one or a combination of cytokines could induce CAR expression on such cells. The following cytokines were tested in one, two, and four-day cultures, either alone or in combination: SF, FL, IL-6, IL-3, IL-11, GM-CSF, and Epo. No cytokine or combination thereof—33 conditions were tested—could be identified that showed a superior effect on CAR expression (data not shown). All subsequent cell cultures were therefore performed in serum-free medium, supplemented with SF, FL, IL-6, and Epo, a culture condition demonstrated to maintain the most

primitive hematopoietic cells [1, 24, 25]. Over a seven-day culture period, the number of CD34<sup>+</sup> cells that express CAR stays nearly constant (Fig. 5). However, the number of CD34<sup>+</sup> cells that express CAR increases dramatically with time. Thus, as CD34<sup>+</sup> cells lose CD34 expression, they acquire CAR. This result, together with the phenotypic analysis and functional CFC data, suggest that expression with CAR in hematopoietic cells is related to myeloid and erythroid differentiation.

## DISCUSSION

Adenovirus-mediated gene transfer is highly efficient in permissive cells, such as HeLa cells, or nonpermissive cells stably infected with the gene encoding CAR [11, 14]. In contrast, we show here that the transduction of genes into primitive CD34<sup>+</sup> hematopoietic cells by an adenovirus construct is not very effective. In our hands, only 15%-20% of CD34<sup>+</sup> cells exposed for 20 h to adenovirus were transduced, a result that directly correlated with the level of CAR expressed on the cell surface. However, CAR expression was found to be associated with cellular differentiation. These results predict very low adenovirus-mediated gene transfer into immature long-term repopulating hematopoietic stem cells (HSCs). Indeed, in one experiment in which purified CD34<sup>+</sup>CD38<sup>-</sup> cells were exposed to AdGFP for 20 h of the 24 h in culture, only 2% gene transfer efficiency could be demonstrated (data not shown). These results appear to contrast with a previous report showing that quiescent CD34<sup>+</sup>CD38<sup>-</sup> cells were GFP<sup>+</sup> after exposure to an adenovirus GFP construct [2]. Several reasons may account for this difference: first, the post-infection time allowing for gene expression (24 h in our experiment versus 48 h), and second, the starting population that was infected. We infected purified CD34<sup>+</sup>CD38<sup>-</sup> cells, whereas Neering *et al* used total CD34<sup>+</sup> cells and analyzed the proportion of transduced CD34<sup>+</sup>CD38<sup>-</sup> cells by FACS. It is possible that there are accessory cells present in the CD34<sup>+</sup> cell population that facilitate gene transfer into other cells. The mechanism by which this occurs is unclear, but it is tempting to speculate that these cells produce certain cytokines that upregulate cell surface molecules, as yet unidentified, that are important for adenoviral infections in primitive hematopoietic cells. Interestingly, although adenoviral gene transfer into human primitive cells is inefficient at best, murine long-term repopulating HSCs are quite efficiently transducible with the same construct (unpublished data, 1999).

Several approaches have been taken to improve adenovirus infection of otherwise nonpermissive cells, such as modulating the viral surface structures with which the virus may attach to the target cells and the use of agents to facilitate the virus-target cell binding [26-28]. Our approach, i.e., trying to induce CAR expression on hematopoietic progenitor cells, has so far been unsuccessful. The cytokines we tested were chosen based on previous studies describing their (relative) beneficial effect on CD34<sup>+</sup> cells in maintaining NOD/SCID mouse repopulating ability [1, 29, 30]. Because our goal was not only to induce CAR, but also to maintain phenotype/function, the cytokines that were tested were limited to SF, FL, IL-6, IL-3, IL-11, GM-CSF, and Epo. However, studies with other cell types may point us towards examining other cytokines, not usually thought of in relation to culturing CD34<sup>+</sup> cells. In this regard it is of interest that IL-2 was found to induce the expression of the fiber receptor on the cell surface of lymphocytes, whereas this receptor was undetectable in noncultured, freshly isolated lymphocytes [31]. Whether this receptor was indeed CAR needs to be verified, or, for example the  $\alpha_4\beta_2$  integrin, as others have shown to be involved in adenovirus binding to human monocyte cells [32]. Interestingly, a small subset of CD34<sup>+</sup> cells do express this integrin [33] and may provide a tool to improve adenovirus-mediated gene transfer into hematopoietic progenitor cells.

In conclusion, our results clearly demonstrate that CAR is expressed on hematopoietic cells and that its expression is directly related to the susceptibility of these cells to adenoviral gene transfer. We also demonstrate that the majority of CAR<sup>+</sup> hematopoietic cells are lineage-committed cells and not the more primitive CD34<sup>+</sup> progenitor cells. Our findings, along with those of others [2], that high MOIs are needed to successfully infect hematopoietic cells with adenovirus, suggest that at low MOI, adenovirus may provide a good vehicle for oncolytic therapy in cancer.

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